CELL SUSPENSION CULTURE OF *AQUILARIA MALACCENSIS* LAMK.

SITI ZALIKHA BINTI MOHD RIDZUAN JAMIL

This report is submitted in fulfillment of requirement for degree of

Bachelor of Science with Honours

(Plant Resource Science and Management)

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2013

APPROVAL SHEET

Name of candidate : Siti Zalikha Binti Mohd Ridzuan Jamil

Title of dissertation : Cell Suspension Culture of *Aquilaria malaccensis* Lamk.

_____

(Prof. Dr Hamsawi bin Sani)

Supervisor

_____

(Prof. Dr Sepiah Binti Muid)

Co-supervisor

_____

(Dr Rebicca Edward)

Coordinator of Plant Resource Science and Management Programme

Department of Plant Science and Environmental Ecology

Faculty of Resource Science amd Technology

Universiti Malaysia Sarawak (UNIMAS)

DECLARATION

I hereby declare that this Final Year Project 2013 is based on my original work except for quotations and citations, which have been duly declared that it has not been or concurrently submitted for any degrees at UNIMAS or other institutions of high education.

_____

Siti Zalikha Binti Mohd Ridzuan Jamil

Plant Resource Science and Management

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak (UNIMAS)

ACKNOWLEDGEMENTS

First and foremost, I would like to express my humble thanks and grateful to ALLAH S.W.T. for the strength, inspiration and encouragement given to me throughout the completion of this thesis. A lot of experiences and knowledge were gained along the way. I wish to express my sincere appreciation to my supervisors, Prof. Dr Hamsawi bin Sani and other lecturers for their critics, advices, motivation and input of ideas, relentless support, guidance and endless encouragement throughout my study in plant tissue culture.

I would like to express my appreciation to postgraduate student, Mr. Zul Helmey Mohamad Sabdin for guiding and assisting me throughout this research. My sincere appreciation also extends to all my fellow colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Thank you for the time sacrificed to accompany me. And last but not least, special thanks to my beloved family, Mohd Ridzuan Jamil Raj Bin Abdullah, Sariah Binti Banau, Siti Zahirah Mohd Ridzuan and Mohd Hafiz, for their encouragement, love and prayers from the beginning of my study.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
2,4- D	2, 4-dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
NAA	1-naphthaleneacetic acid
CITES	Convention Trade in Endangered Species of Wild Fauna and Flora
HCl	Hydrochloric acid
IUCN	International Union Conservation Nature
KOH	Potassium hydroxide
MS	Murashige and Skoog's medium
PGR's	Plant Growth Regulators
PTC	Plant Tissue Culture

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

Cell Suspension Culture of *Aquilaria malaccensis* Lamk.

Siti Zalikha Binti Mohd Ridzuan Jamil

Plant Resource Science and Management
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

The genus *Aquilaria* as it is commonly known as gaharu is a smooth bark tree with aromatic smell. It is under the Thymelaeceae family. The most common species of *Aquilaria* are *A. malaccensis, A. agallocha, A. microcarpa* and *A. beccariana*. It produces resin which has aromatic smell. It will produce gaharu oil when it is infected by the fungi. The economic importance of production of gaharu is medicine, perfume and incense. Much interest has shown to produce gaharu in vitro through tissue culture as the wild supply of the species has been decreased. Nowadays, tissue culture can produce oil of gaharu without planting the tree. In this study, callus of *Aquilaria malaccensis* was induced for the suspension culture. In the induction of callus, 2.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (BAP) was used as the plant growth regulator added to the Murashige and Skoog medium. The growth performance of fully developed callus can be obtained after four weeks of cultured. Cell suspension culture was done with different effect of sucrose and casein hydrolysate concentration. In sucrose effect, 40.0 g/L of sucrose in liquid MS medium enhance the growth of callus while in casein hydrolysate effect, 0.2 g/L, 0.3 g/L and 0.4 g/L could enhance the growth of callus with no difference. Therefore, further research need to be done for promoting the growth of callus.

Keywords: *Aquilaria malaccensis*, callus, suspension culture, sucrose, casein hydrolysate

ABSTRAK

Genus Aquilaria atau dikenali sebagai gaharu, adalah pokok yang mempunyai kulit licin dan bau aromatik. Ia adalah di bawah Famili Thymelaeceae. Spesies yang biasa dikenali adalah A. malaccensis, A. agallocha, A. microcarpa dan A. beccariana. Ia menghasilkan resin yang mempunyai bau aromatik. Ia akan menghasilkan minyak gaharu apabila ia dijangkiti oleh kulat. Minyak gaharu banayak digunakan sebagai ubatan, minyak wangi dan kemenyan. Penghasilan gaharu in-vitro mendapat perhatian ramai kerana spesis ini semakin berkurangan. Kini, kultur tisu boleh menghasilkan minyak gaharu tanpa menanam pokok. Dalam kajian ini, kalus Aquilaria malaccensis telah diinduksi untuk ampaian sel. Dalam induksi kalus, 2.0 mg / L asid 2,4-Dichlorophenoxyacetic (2,4-D) dan 0.5 mg / L 6-benzil amino purina (BAP) telah digunakan sebagai pembantu pertumbuhan tumbuhan ditambah kepada media Murashige dan Skoog. Prestasi pertumbuhan kalus sepenuhnya boleh diperolehi selepas empat minggu kultur. Ampaian sel dilakukan dengan kesan dan kepekatan berbeza sukrosadan kasein hidrolisat. Pada hakikatnya sukrosa, 40.0 g / L sukrosa dalam cecair MS medium meningkatkan pertumbuhan kalus manakala di kasein kesan hidrolisat, 0.2 g / L, 0.3 g / L dan 0.4 g / L boleh meningkatkan pertumbuhan kalus dengan perbezaan. Oleh itu, kajian lanjut perlu dilakukan untuk menggalakkan pertumbuhan kalus.

***Kata kunci**: Aquilaria malaccensis, kalus, ampaian sel, sukrosa, kasein hidrolisat*

1.0 INTRODUCTION

1.1 Research Background

Agarwood or commercially known as gaharu is the resin-impregnated heartwood of *Aquilaria* species of the family Thymelacaceae. Gaharu produces aromatic smell when the wood is burnt. The wood of *Aquilaria* has fragrant smell that has been in the form of wood chips, powder and oil since long time ago for the use in religion and for medicinal and aromatic products (Zich & Compton, 2001). Gaharu is widely known as an economic importance throughout the world.

In the genus of *Aquilaria*, there are fifteen known species where it is found that eight of them produce gaharu (Nurul Ain *et al.*, 2011). According to Barden *et al*. (2000), different species of gaharu has different characteristics. Each species will be graded according to their characteristics. The characteristics that will be graded are the origin of gaharu, fragrance strength and longevity, wood density, product purity, resin content, colour, and the size of traded form.

In this study, there are some challenges that will be faced. According to Okudera and Ito (2009), *Aquilaria* species is facing problems on the shortage of resources in tropical rainforest area where it is listed as endangered species in Apendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora. *Aquilaria malaccensis* Lamk. Also listed in IUCN Red List as endangered

species. Therefore, there is a need to preserve the *Aquilaria* species from extinction. Apart from that, uncontrolled felling of trees also contributes to the shortage of trees.

Since gaharu has high economical value for medicine, perfume making and incense, it is important to have knowledge on the alternative means to produce gaharu, especially through suspension culture. Hence, the production of high quality of gaharu can be enhanced. Large volume of calli will be produced to supply enough material for extraction. Suspension culture is an alternative method to produce callus. Once the proper protocol is achieved, the production of callus will be enhanced by the use of bioreactor.

1.2 Objectives

The objectives of this study are:

1) To establish cell suspension culture of *Aquilaria malacensis* using Murashige and Skoog's medium
2) To determine the effect of sucrose on callus growth and development
3) To determine the effect of elicitor for secondary metabolite using Casein hydrolysate.

2.0 LITERATURE REVIEW

2.1 Thymelaecaceae family

Thymelaecaceae family is found in the tropical region of Asia and to some extend in the subtropical regions of Africa and Australia. Under this family, it has two genus which is genus *Aquilaria* and genus *Daphne*. *Aquilaria* or also known as Eaglewood tree is important in economic value (Corner, 1988). *Aquilaria* species is the main source of Gaharu (Soehartono & Newton, 2001) which is the formation of fragrant resin in the heartwood. *Aquilaria* is a hardwood tree but it is of little use as timber because the wood is light, soft and very perishable (Whitmore, 1972).

The wood of *Aquilaria* species is white or brownish yellow in colour with hard and light texture (Nor Ilia Anisa, 2008). Under genus *Aquilaria*, there are 12 species of *Aquilaria*. Some of them are *A. microcarpa*, *A. beccariana*, *A. hirta* and *A. malaccensis*. The leaves of this tree are alternate, 5-11cm long and 2-4 cm broad, with a short acuminate apex and an entire margin. The flowers is white in colour (Oyen & Nguyen, 1999). Based on the results of Fourth National Forest Inventory carried out from 2002 to 2004, *Aquilaria* trees was estimated in total of 3.55 million trees with a total volume of 1.79 million cubic meters in natural forests. From the total number of trees, 95% of them having diameter at breast height (DBH) ranging from 15 cm to 45 cm (The Forestry Department, 2007).

A. malaccensis will produce seeds after seven to nine years and the seeds are able to germinate within 16 to 63 days. The type of germination of *A. malaccensis* is epigeal and hypogeal. Gaharu is well known to produce resin but not all mature trees produce the resin and it depends on the species itself (Chua, 2008). It is mostly known nowadays because of its wood that produces pleasant smell. This wood is usually found only in dying trees that is caused by a disease. The characteristics of the wood are soft, pale and odourless and it has a dark wood which form lumps. These lumps are called agarwood which has economic value (Corner, 1988). Resin of *Aquilaria* spp. produce when the trees are infected or wounded where it triggered by mechanical wounding and disease infection. Resin production is one type of plant defense response in *Aquilaria* trees (Okudera and Ito, 2009). Resin of *Aquilaria* species produce in the resin canal. Resin canal is located at the heartwood of phloem where it act as defense mechanism in the tree. When the tree is infected by the fungus, it will produce aromatic resin and this resin usually produced several milimetres above from the hole drilled on longitudinal section and contact with the hole on tangential direction (Tabata *et al*., 2003).

Resin formation in *Aquilaria* spp. contain secondary compound which is phytoalexin compound that acts as defense mechanism (Novriyanti *et al*., 2011). Based on the previous research done by Novriyanti *et al*. (2011), *Fusarium* sp. is used to determine the phytoalexin compund in resin formation. Two different localities of *Fusarium* sp. which are from Tamiang Layang (Central Kalimantan) and Maluku.

Inoculation of *Fusarium* sp. from Tamiang Layang produced high concentration of phytoalexin while inoculation of *Fusarium* sp. from Maluku produce high concentration of odorants-character compounds.

2.1.1 Taxonomic classification of *Aquilaria malaccensis*

Aquilaria malaccensis Lamk. is the name given in 1783. It is a Latin word which means 'from Malacca', a place in Peninsular Malaysia. According to the IUCN Red List Of Threatened Species ™ (2012), the botanical classification of gaharu is:

Class: Magnoliopsida

Order: Myrtales

Family: Thymelaeaceae

Scientific name: *Aquilaria malaccensis* Lamk.

Common name/s: Agarwood, Aloewood, Eaglewood, Lign-aloes

2.1.2 *Aquilaria* sp. distribution

Aquilaria malaccensis is widely distributed in South and Southeast Asia. Oldfield *et al.* (1998) has listed 10 countries where the *A. malaccensis* was found, which are Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand. This species is also included in the World List of Threatened Trees. *Aquilaria* species usually can grow in different habitats. It can grow on rocky, sandy or calcareous, well drained slopes and ridges and swamps. This species can grow at altitudes between 0 to 850 m with temperature of 20- 22º C (Wiriadinata, 1995). In Malaysia, this species is usually found mainly in plains, hill slopes and can grow up to 750 m in lowland and hill dipterocarp forests (Jantan, 1990). While in Sarawak, it is hard to find *A. malaccensis* compared to the other species (Tawan, 2004).

2.1.3 Economic importance of *Aquilaria malaccensis*

2.1.3.1 Medicine

Gaharu has medicinal value. Gaharu helps to remove the bad chi or energy from the body, which promotes the circulation of blood flow. High grade gaharu powder is prescribed in Chinese medicine and used in the production of pharmaceutical tinctures (Yaacob, 1999). According to Okugawa *et al.* (1996), gaharu also can be used to treat stomach ache and sedative, and has been proved having

antibiotic, anti-tumor and anticancer properties. Besides that, gaharu have been extracted to form ointment for smallpox and abdominal pains. It also have been prescribed for heart palpitations and as a tonic during pregnancy, after childbirth and disease of female genital organs (Chakrabarty *et al*., 1994).

Agarwood is also known in Japan and it has been used widely in their cultural, religion and for medicinal purposes over hundred years ago. According to Compton and Ishihara (2004), Japanese recognized agarwood as *jin-koh*. *Jin-koh* was a symbol of power and wealth in feudal Japan. It is used as sedative and benzene extract for reduction of spontaneous motility in mice (Okugawa *et al*., 1996).

2.1.3.2 Perfume

In India, several types of grades of gaharu are distilled separately before blending to produce "minyak attar". This oil is a water-based perfume containing gaharu oil, which is traditionally used by Muslims lace prayer clothes (Yaacob, 1999). Gaharu perfume is seldom pure gaharu oil, but instead it uses alcoholic or non alcoholic carrier. Usually, the synthetic or blend of oils is considered as the cheapest perfume of gaharu which has different qualities and fragrances. The essence of gaharu has been recently used as fragrances in soaps and shampoo (Chakrabarty *et al*., 1994).

2.1.3.3 Incense

Gaharu powder and dust cannot be burned directly in the incense holders. Mostly, it has been used to make incense sticks or coils for house fragrance. Gaharu incense is burned and it will produce pleasant smell of aromatheraphy. The aromatic smell is 100% pure natural smell. Different parts of the woods of Gaharu produce different aromatic smell . No chemicals or any artificial perfumes are added, and this perfume is safe to use (Yaacob, 1999). In Japan, incense was used as incense ceremony due to its fragrant smell (Okudera and Ito, 2009). The agarwood contains sesquiterpenes and chromones which are complex compounds that produce fragrance (Suwardi *et al*., 2009). The price of gaharu was sold from US $60/kg up to US $500/kg according to the quality itself. Therefore, the high quality of gaharu will be used to make incense whereas the low quality of gaharu will be extracted to get the oil used for religous ceremonies, cosmetics and perfume (Paoli *et al*., 2001).

2.2 *In- vitro* culture of plant species

In- vitro culture is a technique to propagate plant using the other parts of plant. The small parts of the plant are cultured aseptically on nutrient medium where the nutrients will stimulate the growth and development of tissues (Bonga, 1982). Tissue culture has been used in research study to investigate the totipotency and the importance of hormones in cytodifferentiation and organogenesis in the past (Mineo,

1990). Moreover, by tissue culture technique, high quality of seedlings can be produced for plantation in the future where it reduces the juvenile phase of the growth and it takes a short period of time. Besides that, through tissue culture, new plants can be established with true-to-type plant and free from pest and diseases (Azwin *et al*., 2006).

In-vitro culture is one of the important methods for farmers to increase mass production and to produce "virus-free" stock of plant that are highly- resistant to diseases. Moreover, through tissue culture, secondary products can be induced (Bachraz, 1998). All types of plants are possible to produce plantlets from the explants or callus, therefore protocols in micropropagation are available for a wide range of species (Brown and Thorpe, 1995).

2.2.1 Direct and indirect organogenesis of *Aquilaria* spp.

Tissue culture techinque is one of the reliable methods to meet the demand supply of seedlings. This demand increases due to its low viability, low germination rate, slow rate of rooting, long life-cycle and the production of seed are very rare (Salahbiah *et al*., 2006). The formation of shoot for clonal propagation is one of believed techniques where it prevents somaclonal variations in the cell cultures (Hedayat *et al*., 2009). According to He, Qi & Hu (2005), direct organogenesis of shoots on MS medium supplemented with high concentration of BAP inhibits the

growth of shoots while low concentration of BAP led to normal development of shoots. Another research done by Mathius *et al.* (2008), the MS medium that was supplemented with 3 mg/L of IBA is the best concentration of PGR for the plantlet growth after the micrografting of *A. malaccensis.* While for the growth of shoots of *A. hirta* was best in MS medium supplemented with 0.1 mg/L BAP. The increase in concentration of BAP could decrease the number of shoots produced (Nor Hasnida *et al*., 2011).

Callus culture is actively dividing cells, therefore, different concentration of hormones applied helps in promoting the growth of callus (Pierik, 1997). The explants that will develop into friable or embryonic callus is also known as indirect organogenesis (Hartmann *et al*., 1990). The most widely used auxins for generating callus induction in plant species were 2,4-D. Based on previous research by Pal *et al*. (2006), induction of callus from the hypocotyl part and cotyledons part was succesful using 2,4-D.

Research done by Saikia *et al*. (2013), callus induction using young leaf and explant nodes of *A. malaccensis* grow well on MS medium which are supplemented with plant hormone regulator at high concentration of auxin and low concentration of cytokinin. The best combination of concentration of 2,4-D and BAP concentration that was recorded before was 2.0 mg/L and 0.5 mg/L respectively. They found that 2.0 mg/L 2,4-D has formed the highest number of callus and the highest percentage of callus (Saensouk, 2011). Research done by Zul Helmey, (2010) and Zul Helmey *et al*.

(2011a), 2.0 mg/L 2,4-D and 0.5 mg/L BAP is the best combinations to induce callus through indirect organogenesis.

2.3 Cell suspension culture

Suspension culture is a liquid medium where some of cultures can grow better in it rather than using solid agar medium. This culture needs to be agitated using rotater or shaker which gives oxygen to make sure the cultures can grows callus tissue. Moreover, it will prevent the formation of plantlet but allow the callus to grow more bigger (Kyte and Kleyn, 1996).

There are some benefits using suspension culture to produce better culture. Suspension culture can produce only single cell origin from the somatic embryos and these cells can be used for transferring genes to the other cells passing through particle bombardment (Iantcheva *et al*., 2006). The multicellular callus cells aggregate in high percentage and the present of enzymes in the liquid media helps to break down the clumps of cells (Molnar *et al*., 2011). Suspension culture also has great advantages such as reducing time of production in culture, producing high yield of plant species, and controlling the changes in nature and plant supply (Nhut *et al*., 2006).

According to Linden (n.d), plant tissue culture production can be developed using chemicals that acts as secondary metabolites. Based on the previous research done according by Linden (n.d), suspension cultures can produce useful compounds. Some of the species that produce useful compounds using suspension culture method are *Thalictrum minus* (stomach ache and antibacterial berberine), *Catharanthus roseus* (antihypertension) and *Stilozobium hassjo* (antiparkinson drug).

To produce good cell cultures, the density of the cell cultures is necessary because it is one of the main factors that trigger the biomass accumulation and the production of secondary metabolites (Norazlina et al., 2006).

Research done by Rusli *et al*. (2007), young leaves of *A. malaccensis* could be induced in cell suspension culture. The young leaves were cultured on MS medium supplemented with 2,4-D and BAP as a plant regulator. Friable callus could be induced within four weeks and it is suitable for cell suspension cultures and the production of secondary metabolites. Shu *et al*. (2005) has done cell suspension culture of A. sinensis from seeds. Tissue cell of the seed are cultured on MS medium supplemented with 1.1 µM NAA, 2.2 µM BA and 15 g/L sucrose. Cell suspension culture was established in liquid MS medium consisting of 10.7 µM NAA and 2.2 µM BA.

2.3.1 Sucrose

Sugar plays important roles in plants, in which it functions as carbohydrate supply (Nhut *et al*., 2006). Carbohydrates are known as carbon source which provide energy to the cell cultures (Koch, 1996). Sucrose is one of the common sources that has been used in tissue culture. According to Nhut *et al*. (2006), during the sterilization of the medium at 121 °C, the sucrose that was added to the media will undergo hydrolysis which converts it into glucose and fructose. Due to hydrolysis, the sucrose will change the concentration of sugar and osmotic pressure and this causes the process of changing to suit the different conditions will be slow.

In tissue cultures, the growth of cultures inhibits the formation of chlorophyll and the process of photosynthesis, therefore sucrose will support the growth of cell cultures (Edelman and Hanson, 1972). The effect of sucrose in suspension culture will enhance the callus cell and increase the number of cells (Sakuta *et al*., 2006). Research done by Saikia *et al*. (2012), the best callus growth is at the optimum concentration of sucrose which is 40g/L of sucrose. Sucrose concentration gives bigger impacts to the callus induction, where the concentration above the optimum level decreases the callus production.